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# E1210, a New Broad-Spectrum Antifungal, Suppresses *Candida albicans* Hyphal Growth through Inhibition of Glycosylphosphatidylinositol Biosynthesis

**Nao-aki Watanabe, Mamiko Miyazaki, Takaaki Horii, Koji Sagane, Kappei Tsukahara, and Katsura Hata**

Next Generation Systems Core Function Unit, Eisai Product Creation Systems, Eisai Co., Ltd., Tsukuba, Ibaraki, Japan

**Continued research toward the development of new antifungals that act via inhibition of glycosylphosphatidylinositol (GPI) biosynthesis led to the design of E1210. In this study, we assessed the selectivity of the inhibitory activity of E1210 against *Candida albicans GWT1* (Orf19.6884) protein, *Aspergillus fumigatus GWT1* (AFUA_1G14870) protein, and human *PIG-W* protein, which can catalyze the inositol acylation of GPI early in the GPI biosynthesis pathway, and then we assessed the effects of E1210 on key *C. albicans* virulence factors. E1210 inhibited the inositol acylation activity of *C. albicans* Gwt1p and *A. fumigatus* Gwt1p with 50% inhibitory concentrations ($IC_{50}$s) of 0.3 to 0.6 $\mu$M but had no inhibitory activity against human Pig-Wp even at concentrations as high as 100 $\mu$M. To confirm the inhibition of fungal GPI biosynthesis, expression of *ALS1* protein, a GPI-anchored protein, on the surfaces of *C. albicans* cells treated with E1210 was studied and shown to be significantly lower than that on untreated cells. However, the *ALS1* protein levels in the crude extract and the *RHO1* protein levels on the cell surface were found to be almost the same. Furthermore, E1210 inhibited germ tube formation, adherence to polystyrene surfaces, and biofilm formation of *C. albicans* at concentrations above its MIC. These results suggested that E1210 selectively inhibited inositol acylation of fungus-specific GPI which would be catalyzed by Gwt1p, leading to the inhibition of GPI-anchored protein maturation, and also that E1210 suppressed the expression of some important virulence factors of *C. albicans*, through its GPI biosynthesis inhibition.**

The incidence of life-threatening fungal infections has increased steadily over the past 2 decades as a result of an increase in the number of susceptible immunosuppressed patients (39, 68). However, there are a limited number of antifungals available that can safely and effectively treat serious invasive fungal infections in humans. Drugs available for human invasive fungal infections are represented principally by four classes of compounds—polyenes, fluorinated pyrimidines, azoles, and echinocandins—but they each have limited usefulness because of their limited spectrum of antifungal activity, adverse effects, drug-drug interactions, variable pharmacokinetics (often requiring therapeutic drug monitoring), and/or only one type of formulation (12). Resistance is also becoming an increasing problem, particularly among members of the azole class (33, 44, 45, 47, 66). Therefore, new antifungal drugs with novel mechanisms of action which show no cross-resistance to existing antifungals are desirable for the treatment of serious invasive fungal infections.

We have also focused our attention on ways to interfere with the expression of virulence factors that are associated with the establishment of fungal infections in order to design an optimal novel antifungal agent. Microorganisms must first attach to host cell surfaces in order to establish infections; this is followed by colonization and replication on these host mucosal or endothelial surfaces and then by pathogen penetration across one or more mucosal barriers or by systemic dissemination through the intravascular route. Some of the fungal ligands (adhesins) responsible for the adhesion step are derived from glycosylphosphatidylinositol (GPI)-anchored proteins. For example, *Candida albicans*, which is one of the most common fungal pathogens encountered in the clinical setting, is estimated to possess 115 GPI-anchored proteins (51). Some GPI-anchored proteins are known to be transported to the cell wall and then function as adhesins (7). The Als protein family members are typical GPI-anchored proteins. Inhibition of GPI-anchored-protein maturation is a promising therapeutic goal for the treatment of fungal infections.

In the course of screening compounds that inhibit the cell wall assembly of GPI-anchored proteins, we discovered the *GWT1* gene (63), which encodes an inositol acyltransferase found early in the GPI biosynthesis pathway (36, 64), and this led to the creation of an inhibitor of this enzyme, 1-(4-butylbenzyl)isoquinoline (BIQ) (63). In our efforts to improve the efficacy of this prototype-inhibitor BIQ (38, 59), we next developed E1210, 3-(3-{4-[(pyridin-2-yloxy)methyl]benzyl}isoxazol-5-yl)pyridin-2-amine (34). This compound showed potent *in vitro* antifungal activity against a broad range of pathogenic fungi, including *Candida* spp., *Aspergillus* spp., and other molds, such as *Fusarium* and *Scedosporium* spp., although its action was fungistatic (34), and subsequently showed high therapeutic efficacy in several representative *in vivo* models of invasive fungal infections (19).

In this study, we investigated the inhibitory activity of E1210 on the inositol acylation of GPI, and we showed that the mechanism of action of E1210, inositol acyltransferase inhibition in the GPI biosynthesis pathway, involved selective inhibition of fungal but not human enzymes. In addition, we investigated the effects of E1210 on the expression of the *ALS1* protein (Als1p), a GPI-

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Address correspondence to Nao-aki Watanabe, n3-watanabe@hhc.eisai.co.jp.

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anchored protein, on the cell surface of *C. albicans* and on virulence factors of *C. albicans*: germ tube formation, adherence, and biofilm formation. There is a possibility that the *GWT1* protein (Gwt1p) inhibitors might prove useful for the treatment of infections due to other microbes.

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## MATERIALS AND METHODS

**Antifungals.** E1210 was synthesized by Eisai Co. (Tokyo, Japan). Fluconazole was extracted from fluconazole tablets (Pfizer Japan Inc., Tokyo, Japan), and micafungin was obtained in vials (Astellas Pharma Inc., Tokyo, Japan). Amphotericin B was purchased from Sigma-Aldrich Co. (St. Louis, MO). Compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with an appropriate medium to yield a DMSO concentration of 1%. All of the solutions were prepared on the day of use.

**Cloning of fungal *GWT1* and human *PIG-W* genes.** Cloning of *GWT1* genes from *C. albicans* and *A. fumigatus* was described in our previous report (63). Briefly, the *C. albicans GWT1* (*CaGWT1*) gene was isolated from a clinical isolate in our laboratory. The amino acid sequence of the *CaGWT1* product is 100% identical to that of the *GWT1* protein from *C. albicans* SC5314 strain (XP_712813.1; locus tag Cao19.6884) in the NCBI database. *A. fumigatus GWT1* (*AfGWT1*) was isolated from a commercial cDNA library purchased from Stratagene Corp. (La Jolla, CA). We found one amino acid substitution, I197T, between our *AfGWT1* product and AFUA_1G14870 gene product in the NCBI database (accession no. XP_752856.1). DNA fragments containing the human *PIG-W* gene were amplified from human genomic DNA using primers hGW044F (GCCCAAAGTTCTGCTCTGAAATCTAGT) and hGW043R (ATGGTT AACACTGTTGCCAAAAGCACA) and then cloned into pT7-Blue vector. The DNA sequence of human *PIG-W* which we cloned was identical to that of the *PIG-W* gene deposited in the NCBI database (NM_178517.3).

**Plasmid construction.** *CaGWT1* was inserted into the multiple-copy plasmid YEp352GAPII and the single-copy plasmid pRS316 (42), and the resultant newly constructed plasmids were designated YEp-Ca and pRS-Ca, respectively. Likewise, the *AfGWT1* and human *PIG-W* genes were cloned into plasmids YEp352GAPII and pRS316, and the resultant plasmids were designated YEp-Af, pRS-Af, YEp-hum, and pRS-hum. Plasmid YEp provides high levels of Gwt1p expression and pRS supports lower levels of Gwt1p expression in *Saccharomyces cerevisiae*. Plasmid introduction into the diploid strain WDG2 of *S. cerevisiae* (63) and heterozygous deletion of *GWT1* were performed using a Yeast Maker yeast transformation kit (Clontech Laboratories, Mountain View, CA), and the haploid cells deleted for *GWT1* gene and complemented by orthologous *GWT1* or *PIG-W* protein expression were segregated from the transformed WDG2 cells. We obtained *gwt1* knockout cells complemented by YEp-Ca, YEp-Af, YEp-hum, pRS-Ca, and pRS-Af; however, we found that pRS-hum does not rescue growth defects caused by Gwt1p depletion. *S. cerevisiae* strains used in this study are listed in Table 1.

**Other organism and media.** *C. albicans* strains were provided by the Medical Mycology Research Center, Chiba University (Chiba, Japan), and the Graduate School of Medicine, Gifu University (Gifu, Japan). A total of 36 strains were clinically isolated in Japanese hospitals from 2000 to 2005, and eight strains were isolated from blood, 6 from sputum, 2 from pharyngeal mucosa, 16 from female genitals, and 4 from other clinical sites. Strain IFM49971 was mainly used for each assay described below. *Escherichia coli* IFO12734 and *Staphylococcus aureus* IFO12732 were provided by the Institute for Fermentation (Osaka, Japan).

Synthetic defined medium without uracil [SD(−Ura)] [yeast nitrogen base without amino acids (Becton Dickinson Company, Sparks, MD) supplemented with 0.5% glucose and 0.077% −Ura DO supplement (Clontech Laboratories)] was used for *S. cerevisiae* growth. For *C. albicans*, Sabouraud dextrose broth (SDB; Becton Dickinson Company), YPS agar (1% yeast extract, 2% Bacto peptone, 2% sucrose, and 1.2% Bacto agar), and RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) buffered with 0.165 M 3-morpholinopropane sulfonic acid (MOPS) (pH 7.0) (MOPS–RPMI 1640) were used, and for germ tube formation assay of *C. albicans*, yeast nitrogen base with amino acids supplemented with 0.5% glucose (YNB), Spider medium (31), Lee's medium (28), YPD broth (Becton Dickinson Company) supplemented with 10% fetal calf serum (FCS), and yeast nitrogen base without amino acids but supplemented with 2.5 mM *N*-acetylglucosamine (35) were also used. Mueller-Hinton broth (MHB; Becton Dickinson Company) was used for bacteria.

**TABLE 1** *S. cerevisiae* strains used in this study

| Strain | Genotype |
|---|---|
| W303-1A | *MAT*-**a** *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100* |
| W303-1B | *MAT*-α *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100* |
| W303D | *MAT***a**/α *ade2-1/ade2-1 his3-11/his3-11 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 can1-100/can1-100* |
| WDG2 | *MAT*-**a**/α *ade2-1/ade2-1 his3-11/his3-11 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 can1-100/can1-100 GWT1/gwt1::his5*$^+$ |
| KE666 | *MAT*-α *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100 gwt1::his5*$^+$ [YEp-Ca] |
| KE668 | *MAT*-α *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100 gwt1::his5*$^+$ [YEp-Af] |
| KE249 | *MAT*-α *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100 gwt1::his5*$^+$ [YEp-hum] |
| KE798 | *MAT*-α *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100 gwt1::his5*$^+$ [pRS-Ca] |
| KE802 | *MAT*-α *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100 gwt1::his5*$^+$ [pRS-Af] |

**MIC determinations.** The MICs of all compounds were determined using a broth microdilution method in accordance with the guidelines presented in Clinical and Laboratory Standards Institute (CLSI) documents M27-A3 for yeasts (9) and M07-A7 for bacteria (8). Inoculum sizes were $1 \times 10^3$ for *S. cerevisiae* and *C. albicans* and $5 \times 10^5$ cells/ml for bacteria. After 48 h at 30°C for *S. cerevisiae* and at 35°C for *C. albicans*, or after 18 h at 35°C for bacteria, MICs were read. The MICs of E1210 were defined as the lowest concentration at which a prominent decrease (≥50% of the growth of the compound-free control) in growth turbidity relative to the turbidity of the compound-free control at 660 nm was observed. The MICs of other compounds were read in accordance with the CLSI guidelines.

**Preparation of yeast membrane fractions.** The membrane fractions of *S. cerevisiae GWT1* disruptant harboring YEp-Ca, YEp-Af, or YEp-hum were used as the enzyme sources because the Gwt1p activity in membrane fractions of *C. albicans* and *A. fumigatus* cells was low. Yeast cells grown in SD(−Ura) medium were then harvested by centrifugation, washed twice in 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM $MgCl_2$ (TM buffer), and suspended in TM buffer with 0.1% 2-mercaptoethanol. The cell suspensions were mixed with an equal weight of glass beads (diameter, 0.5 mm), and were homogenized with a cell disruptor [Multi Beads Shocker, model MB601(S); Yasui Kikai Co., Osaka, Japan] operating at 2,500 rpm with 12 cycles of 30-s bursts and 30-s intervals at 0°C. The unbroken cells and glass beads were removed by centrifugation at 3,000 × *g* for 5 min at 4°C. The supernatants were further centrifuged at 20,000 × *g* for 20 min at 4°C, and the pellets were washed with TM buffer. The pellets were resuspended in TM buffer containing 10% glycerol and used as the membrane fraction. The protein concentration was estimated by a Coomassie Plus—The Better Bradford assay kit (Pierce Biotechnology Inc., Rockford, IL) with bovine serum albumin as the standard. The membrane fractions were diluted with TM buffer to 1 mg of protein/ml. They were stored at −80°C until use.

***In vitro* assay for the inositol acylation in GPI biosynthesis.** Inositol acylation was assayed by the method of Umemura et al. (64) with slight

modifications. Membrane fractions (70 μg protein) were incubated in TM buffer containing 2 mM $MnCl_2$, 10 μM nikkomycin Z (Sigma-Aldrich), 21 μg/ml tunicamycin (Sigma-Aldrich), 0.5 mM dithiothreitol, and 0.925 kBq of UDP-[$^{14}$C]-*N*-acetylglucosamine (GlcNAc) (specific activity, 10.7 GBq/mmol; PerkinElmer Life & Analytical Sciences, Boston, MA) for 20 min at 30°C. Then, an excess of unlabeled UDP-GlcNAc (1.94 mM) and compounds were added and further incubated for 20 min at 30°C. Palmitoyl coenzyme A (palmitoyl-CoA) (0.1 mM) (Sigma-Aldrich) was added as an acyl donor, and incubation was continued for 2 h at 30°C. The reaction was stopped by adding 1 ml $CHCl_3$-$CH_3OH$ (1:1, vol/vol). The sample was then incubated at room temperature for about 90 min with shaking (at about 1,400 rpm) and centrifuged at 2,140 × *g* for 5 min at 25°C, after which the supernatant was separated and saved. The pellet was extracted by 0.5 ml $CHCl_3$-$CH_3OH$-$H_2O$ (10:10:3, vol/vol/vol). The pooled lipid extracts were evaporated to dryness under nitrogen gas at 37°C. The lipid was resuspended in 0.5 ml butanol and 0.5 ml $H_2O$ to desalt. After separation by centrifugation, the butanol phase was collected, and then the $H_2O$ phase was re-extracted with 0.5 ml butanol. The butanol phase was pooled, washed once with 1 ml butanol-saturated $H_2O$, and then evaporated to dryness under nitrogen gas at 37°C. The dried lipids were dissolved in 50 μl $CHCl_3$-$CH_3OH$ (2:1, vol/vol) and applied to a Silica Gel 60 plate (Merck KGaA, Darmstadt, Germany). Thin-layer chromatography plates were developed in $CHCl_3$-$CH_3OH$-$H_2O$ (65:25:4, vol/vol/vol) (10). In this solvent system, the order of migration of PI-GlcNAc and PI-GlcN is the reverse of that achieved in the $CHCl_3$–$CH_3OH$–1 M $NH_4OH$ (10:10:3) solvent system which Umemura et al. used (64). Then attached to BAS-MS2040 imaging plates (Fujifilm Co., Tokyo, Japan) for 2 to 5 days for autoradiography. Radioactive products were detected and analyzed with a BAS2500 imaging analyzer (Fujifilm), and the photostimulated luminescence counts of the acylated phosphatidylinositol glucosamine [PI (acyl)-GlcN] zones were quantified. The positions of the radioactive PI (acyl)-GlcN zones were identified as those that were not detected in the absence of palmitoyl-CoA. Each experiment was carried out three times. The inhibition rate (percent) of the inositol acylation of GPI at each compound concentration was calculated, and the mean 50% inhibitory concentration ($IC_{50}$) and 95% confidence interval (CI) were determined.

Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment of radiolabeled lipids was conducted according to the method of Umehara et al. (64).

**Als1p protein levels.** To estimate the amount of Als1p expressed on the cell surface, the compound-treated cells were retained on the filters, and the relative Als1p levels on the cell surface of cells were measured with an enzyme-linked immunosorbent assay (ELISA). *C. albicans* IFM49971 was grown in SDB for 3 days at 25°C without shaking, to obtain a saturated culture containing synchronized cells (20). The culture was centrifuged at 800 × *g* for 5 min at 4°C to pellet the cells, and the pellet was then resuspended in MOPS–RPMI 1640 medium at a concentration of 5 × $10^6$ cells/ml. Since Fu et al. (15) showed that the Als1p expression on the surface of *C. albicans* cells was induced after incubation for 1 h in MOPS–RPMI 1640, *C. albicans* cells were incubated for 1 h at 35°C in MOPS–RPMI 1640 that contained serial fourfold dilutions of E1210, micafungin, or fluconazole on a filter plate (MultiScreen HTS 96-well filtration plate; Millipore Corp., Billerica, MA). Compound-free controls (controls) and *C. albicans*-free controls (blanks) were included. Little change in cell counts was observed after treatment with E1210, micafungin, or fluconazole for 1 h. The concentrations used were 1/4×MIC, 1×MIC, 4×MIC and 16×MIC of each compound. The assay for each concentration for each compound was performed in sextuplicate and repeated three times. After incubation for 1 h, the medium was removed by using a vacuum manifold. The *C. albicans* cells remaining on the filter were washed three times by aspiration with phosphate-buffered saline (PBS) and then were assayed by ELISA. For the ELISA, 4-fold-diluted Block Ace solution (Snow Brand Milk Products Co., Hokkaido, Japan) was used as the blocking agent, anti-Als1 rabbit polyclonal antibody (Sawady Technology Co., Tokyo, Japan) was used as the primary antibody, and anti-rabbit IgG, horseradish peroxidase (HRP)-linked donkey antibody (GE Healthcare UK Ltd., Buckinghamshire, England) was used as the secondary antibody. The amount of Als1p was estimated based on peroxidase activity, which was assayed on filter plate after the addition of 0.1 ml SureBlue Reserve TMB microwell peroxidase substrate (KPL Inc., Gaithersburg, MD) as the substrate. The plates were incubated for 15 min at room temperature, and the reaction was terminated by adding 0.1 ml TMB stop solution (KPL). The reaction mixture was filtered, and the absorbance of each filtrate was measured at 450 nm. The Als1p expression rate (percentage of the control) was then calculated. The expression levels of Rho1p, a membrane protein without a GPI anchor, on the cell surface were likewise evaluated using the ELISA method.

The Als1p levels in crude extracts of *C. albicans* cells were also determined. The cells were incubated for 1 h at 35°C in the presence of E1210. After incubation, the cultures were centrifuged at 1,000 × *g* for 10 min at 4°C and the pellets were suspended in 50 mM potassium phosphate buffer (pH 7.4) containing a protease inhibitor cocktail for fungi (Sigma-Aldrich). The cell suspensions were mixed with an equal weight of glass beads and were homogenized with the cell disruptor. The unbroken cells and glass beads were then removed by centrifugation. The supernatants were obtained as crude extracts. The amount of Als1 protein in the crude extracts was also measured by an ELISA. Anti-Als1 antibody was used as the capture antibody, and anti-*C. albicans*, HRP-conjugated rabbit antibody (ViroStat Inc., Portland, ME) was used as the secondary antibody. An ELISA of each concentration was conducted in duplicate, and the final values were expressed as the means of three determinations. Protein concentration was estimated in order to standardize the amount of Als1p.

**Adherence and germ tube formation in *C. albicans*.** Cells grown in SDB for 3 days at 25°C were resuspended to concentrations of 5 × $10^6$ and 1 × $10^6$ cells/ml in MOPS–RPMI 1640 for the adherence assay and the germ tube formation assay, respectively. *C. albicans* cells were incubated at 35°C in MOPS–RPMI 1640 that contained serial 2-fold dilutions of E1210, fluconazole, micafungin, or amphotericin B. The number of *C. albicans* cells that adhered to the wells of 96-well polystyrene plates was determined using the crystal violet staining assay (1). Compound-free controls (controls) and *C. albicans*-free controls (blanks) were included. The plates were incubated for 1 h and 4 h for the adherence assay and the germ tube formation assay, respectively. The latter assay is based on the observation that *C. albicans* germ tubes adhere to plastic surfaces (1, 62). However, incubation for >1 h in MOPS–RPMI 1640 induces germ tube formation in *C. albicans* cells; it is therefore difficult to compare the inhibitory effects of compounds on *C. albicans* adherence with and without the potential to inhibit germ tube formation in *C. albicans* cells after >1 h incubation. Thus, to evaluate the inhibitory effects of compounds on the adherence of *C. albicans* cells, they were incubated for 1 h, at which time point no distinct cell elongation was apparent (see Fig. 7). In addition, to investigate the adherence potential of *C. albicans* cells that were not cultured in MOPS–RPMI 1640, plates that included only controls and blanks were also prepared and were immediately subjected to the procedures described below.

After incubation, the plates were processed by the procedures of Brayman and Wilks (5). The medium in the plates was discarded by inverting the plates. The remaining *C. albicans* cells in the wells were sterilized by immersion in 70% ethanol, which was discarded, and then 0.2 ml of 0.25% sodium dodecyl sulfate (SDS) (Wako Pure Chemical Industries, Osaka, Japan) was added to each well. The SDS was discarded, and the plates were washed three times by immersion in ion-exchanged water. The *C. albicans* cells that attached to the wells were stained for 20 min at room temperature with 0.1 ml of 0.02% crystal violet (Muto Pure Chemicals Co., Tokyo, Japan) diluted with PBS. The crystal violet solution was removed by inverting the plates. They were then washed three times with water, once with 0.25% SDS, and twice again with water. After the plates were dried, 0.2 ml isopropanol containing 40 mM HCl and 0.05 ml of 0.25% SDS were added to the wells, and the absorbance at 590 nm was

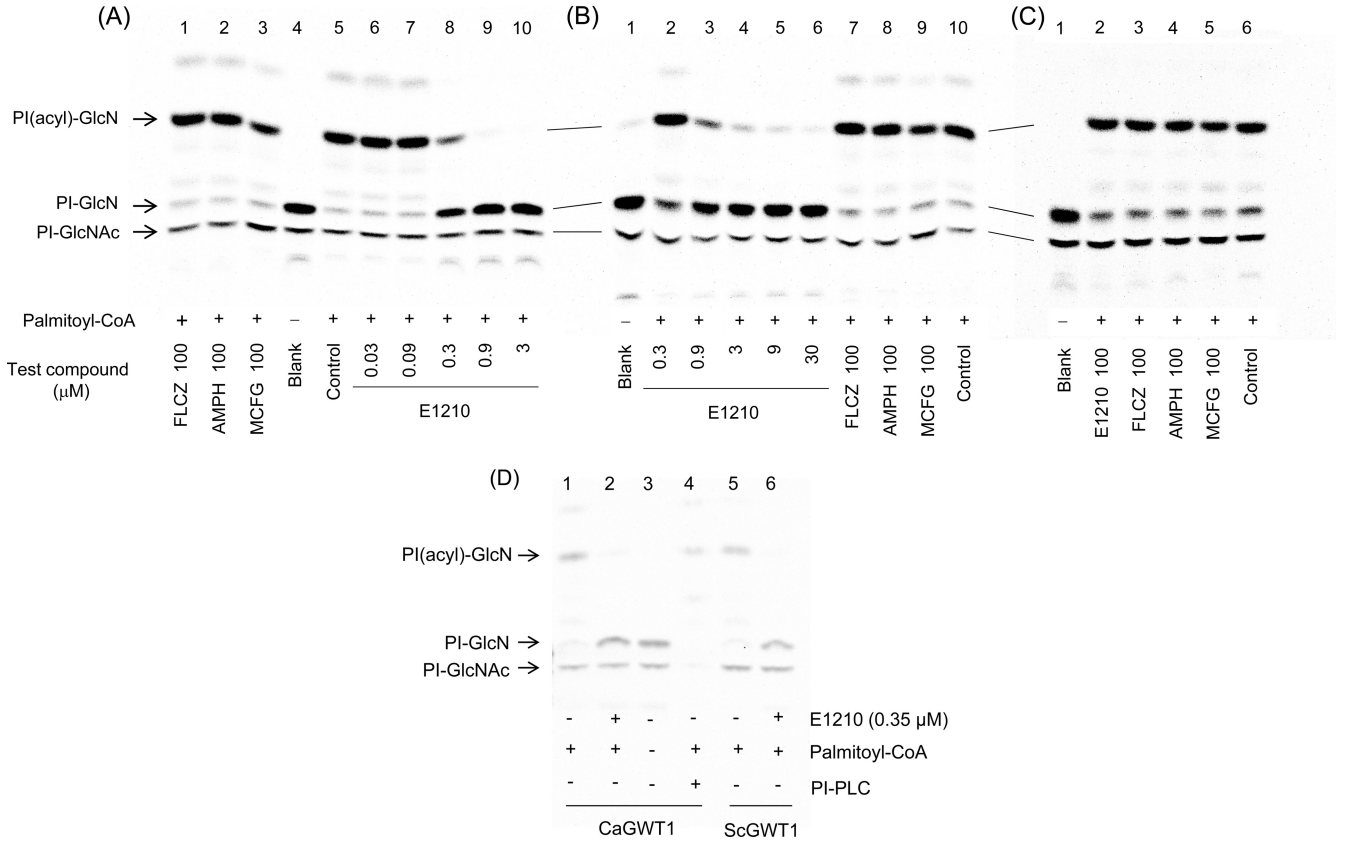


**FIG 1** Autoradiograph of TLC separation of GPI intermediates produced from UDP-[$^{14}$C]GlcNAc in the presence of E1210. The membranes of the *S. cerevisiae GWT1*-disrupted cells overexpressing *C. albicans GWT1* (A), *A. fumigatus GWT1* (B), and human *PIG-W* (C) were assayed for the inositol acylation of GPI in the presence of E1210, fluconazole (FLCZ), amphotericin B (AMPH), and micafungin (MCFG). (D) PI-PLC treatment of radiolabeled lipids produced by membranes of *S. cerevisiae* overexpressing *CaGWT1* and of *S. cerevisiae* overexpressing *ScGWT1*. These images were trimmed from representative autoradiographs of the TLC plates derived from three independent experiments.

measured using a 660-nm reference. The absorbance of each concentration for each compound was measured in duplicate, and the final values were expressed as the means of three determinations. The adherence rate and the germ tube formation rate (percentage of the control) were each calculated, and the mean $IC_{50}$ and 95% CI were calculated. An additional plate was prepared for each assay, and after incubation, 0.05 ml of 3% glutaraldehyde solution (Wako Pure Chemical Industries) was added to the culture wells to fix the cells. After fixation, the *C. albicans* cells were observed by phase-contrast microscopy and the microscopic images of culture wells were each recorded. Effects of E1210 on germ tube formation were also evaluated in YNB, Spider medium, Lee's medium, YPD supplemented with 10% FCS, and yeast nitrogen base without amino acids but supplemented with 2.5 mM *N*-acetylglucosamine.

**Colony morphology.** Cells grown in SDB for 3 days at 25°C were resuspended and diluted to $1 \times 10^4$ cells/ml in physiological saline. Approximately 100 cells were mixed with molten YPS agar that contained serial 2-fold dilutions of E1210 and plated. Plates were incubated for 48 h at 35°C, and colonies were microscopically observed.

**Biofilm formation in *C. albicans*.** The 96-well microtiter plates were pretreated with 0.1 ml of FCS for 24 h at 35°C and washed twice in PBS. *C. albicans* IFM49971 grown in SDB for 3 days at 25°C without shaking was resuspended in MOPS–RPMI 1640 supplemented with 10% FCS. Then, 0.1 ml *C. albicans* cell suspension ($1 \times 10^6$ cells/ml) was added. The plates were incubated for 1 h at 35°C. The medium was discarded by inverting the plates. The plates were washed three times with PBS to remove any unattached planktonic cells. Compound dilutions in MOPS–RPMI 1640 supplemented with 10% FCS were dispensed into a microtiter plate. Compound-free controls (controls) and *C. albicans*-free controls (blanks) were included. After incubation for 24 h at 35°C, the plates were stained using safranin. Safranin is capable of staining exopolymeric material structures (53, 54); therefore, biofilm formation was evaluated by determining the biofilm density after safranin staining. The biofilms were stained for 15 min at room temperature by adding 50 μl of 1% safranin solution (Waldeck GmbH & Co. KG, Münster, Germany). The safranin solution was carefully removed by aspiration, and the wells were washed carefully three times with PBS. Then, 0.2 ml DMSO was added to the wells and mixed gently. Absorbance at 492 nm was measured using a 660-nm reference. Each concentration for each compound was performed in duplicate, and the final values were expressed as the means of three determinations. The biofilm density (% of control) was calculated, and the mean $IC_{50}$ and the 95% CI were also calculated.

MICs for sessile forms (SMICs) of *C. albicans* were also determined according to the method of Jacobson et al. (24). *C. albicans* strains grown in yeast nitrogen base medium were suspended in MOPS–RPMI 1640 at a concentration of $1 \times 10^7$ CFU/ml. Each suspension (0.1 ml) was added to the 96-well microtiter plate and incubated at 35°C. After incubation for 24 h, the suspensions were discarded, and the wells were rinsed three times with PBS and filled with 0.1 ml of compound dilutions in MOPS–RPMI 1640. Microtiter plates were incubated at 35°C for an additional 48 h. Then, media were discarded, and wells were rinsed three times with PBS. The metabolic activity of *C. albicans* cells was measured using the XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide; Sigma-Aldrich] reduction assay, in which a colorimetric change should correlate directly with the metabolic activity of the *C. albi-*

*cans* cells (4, 48). XTT was prepared as a saturated solution at 0.5 mg/ml in PBS. Prior to the assay, stock XTT solution was thawed, and menadione (10 mM in acetone; Sigma-Aldrich) was added to give a final concentration of 25 μM. XTT-menadione solution was then added to the plates, and the plates were incubated in the dark for 30 min at 35°C. The colorimetric change was measured at 492 nm using a 660-nm reference. The SMICs were defined as the lowest concentrations associated with a 50% reduction in absorption compared to the absorption level for the control. MICs for planktonic forms were determined using broth microdilution (9).

**Scanning electron microscopy.** A four-well slide (Sonic Seal Slide Wells; Nalge Nunc International Corp., Rochester, NY) was pretreated with 1 ml of FCS for 24 h at 35°C and washed twice with PBS. Then, the *C. albicans* cells ($1 \times 10^6$ cells) suspended in MOPS–RPMI 1640 were added, and the slide was incubated for 1 h at 35°C. The medium was then discarded, and the slide was washed three times with PBS to remove unattached planktonic cells. Then, 1 ml of MOPS–RPMI 1640 supplemented with 10% FCS containing E1210 (0.008 μg/ml) was added. After incubation for 24 h at 35°C, 1 ml of 2% glutaraldehyde solution was added to the culture wells to fix the cells. After 1 h of fixation, the *C. albicans* cells in biofilms were carefully rinsed four times with PBS and then placed in 1% osmium tetroxide for 1 h. The specimens were subsequently dehydrated in a series of ethanol washes, treated with isoamyl acetate overnight, and finally dried by a critical-point drying method. The specimens were then coated with gold. After the specimens were processed, they were examined under a scanning electron microscope (Type JSM-820; JEOL Ltd., Tokyo, Japan).

**Statistical analysis.** Statistical analyses were performed using the SAS software package, version 8.2 (SAS Institute Japan Ltd., Tokyo, Japan), for the Als1p and Rho1p expression levels.

**Nucleotide sequence accession numbers.** The *CaGWT1* and *AfGWT1* genes were deposited in GenBank under accession numbers AB092481 and AB092482, respectively.

## RESULTS

***In vitro* inositol acylation of GPI.** Figure 1 shows a representative autoradiograph of the thin-layer chromatography (TLC) separation observed in the assay of GPI inositol acylation with E1210 and the other antifungals. Radiolabeled PI (acyl)-GlcN was detected as a product of the inositol acylation of GPI in the membrane fractions of *S. cerevisiae* cells that overexpressed CaGwt1p, AfGwt1p or Pig-Wp. E1210 inhibited the production of PI (acyl)-GlcN by CaGwt1p and AfGwt1p in a concentration-dependent manner, indicating that the compound inhibited an early step of the GPI biosynthesis pathway of *C. albicans* and *A. fumigatus*. The $IC_{50}$s of E1210 for inositol acylation by CaGwt1p and AfGwt1p were 0.27 μM (0.097 μg/ml) (95% CI, 0.18 to 0.39 μM) and 0.60 μM (0.22 μg/ml) (95% CI, 0.24 to 1.5 μM), respectively. In contrast, E1210 had no effect on inositol acylation by human Gwt1p even at 100 μM (36 μg/ml). Fluconazole, amphotericin B, and micafungin had little or no effect on inositol acylation based on all Gwt1ps tested, each yielding $IC_{50}$s of >100 μM.

MICs of E1210 for *ScGWT1*-disrupted *S. cerevisiae* strains harboring plasmids YEp-Ca and pRS-Ca were 0.03 and 0.25 μg/ml, respectively. The MICs for *ScGWT1*-disrupted *S. cerevisiae* strains harboring plasmids YEp-Af and pRS-Af were 0.5 and 2 μg/ml, respectively. Overexpression of fungal *GWT1* proteins reduced the activity of E1210 4- to 8-fold. The MIC of E1210 was >32 μg/ml for *S. cerevisiae* harboring plasmid YEp-hum. No differences in MICs of fluconazole (4 μg/ml), micafungin (0.06 μg/ml), and amphotericin B (4 μg/ml) for strains tested were observed. In addition, E1210 showed no antibacterial activity against *E coli* IFO12734 and *S. aureus* IFO12732 (MICs, >32 μg/ml) since they do not possess any GPI biosynthetic pathway, and the $IC_{50}$ of E1210 for human HK-2 cells was >32 μg/ml (34). These results suggested that E1210 inhibits the inositol acylation of GPI catalyzed by fungal *GWT1* proteins but not by human *PIG-W* protein.

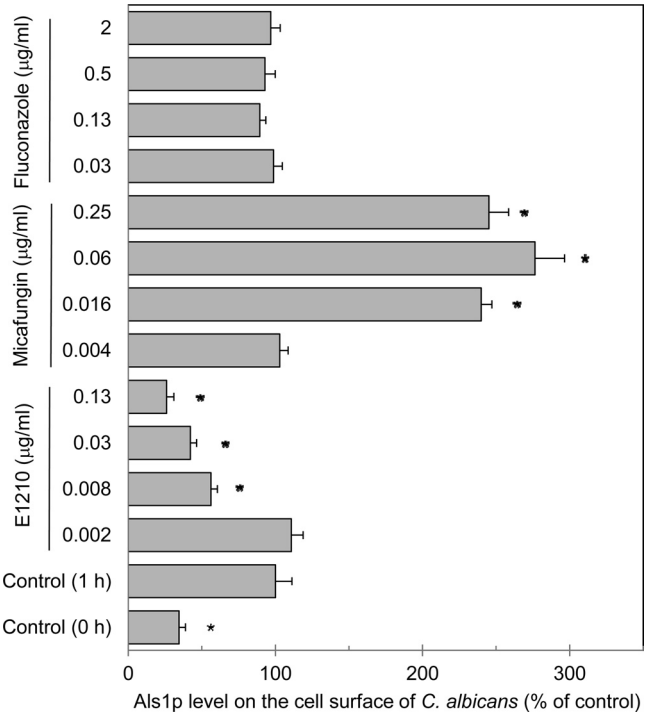


**FIG 2** Effects of E1210 and other antifungals on the expression of Als1 protein on the surface of *C. albicans* IFM49971 cells. The relative amounts of Als1 protein on the cell surface of *C. albicans* treated with each compound in MOPS–RPMI 1640 medium for 1 h were determined on filter plates using ELISAs. Each data point represents the mean ± standard error of the mean (SEM) ($n = 6$). Asterisks indicate a $P$ value of <0.05 compared to control at 1 h, as determined by the Dunnett multiple-comparison test.

**Als1 expression levels.** We evaluated the expression levels of Als1p on the cell surface of *C. albicans* cells treated with E1210 because the compound was expected to inhibit the maturation of GPI-anchored proteins. Als1p expression on the surface of *C. albicans* IFM49971 cells grown for 1 h in MOPS–RPMI 1640 was induced as reported by Fu et al. (15). E1210 suppressed this induction in a concentration-dependent manner (Fig. 2). E1210 showed no suppression of Als1p expression at a concentration of 0.002 μg/ml (1/4×MIC), but the compound suppressed Als1p expression on the cell surface by 44%, 58%, and 74% at concentrations of 0.008 (1×MIC), 0.03 (4×MIC), and 0.13 μg/ml (16×MIC), respectively. In contrast, micafungin further enhanced Als1p expression two- to threefold at concentrations above its MIC (0.016 μg/ml). Fluconazole showed no effects on Als1p expression within the range of concentrations tested (0.03 to 2 μg/ml). The effect of amphotericin B on Als1p expression was not estimated, since amphotericin B reduced the number of viable cells within 1 h.

In addition, the Als1p levels in the crude extracts of *C. albicans* cells treated with E1210 for 1 h were also investigated to exclude the possibility that the reduced levels of Als1p on the cell surface resulted from protein synthesis inhibition potentially due to E1210. E1210 tended to cause increases in Als1p levels in a concentration-dependent manner (Fig. 3), but these increases

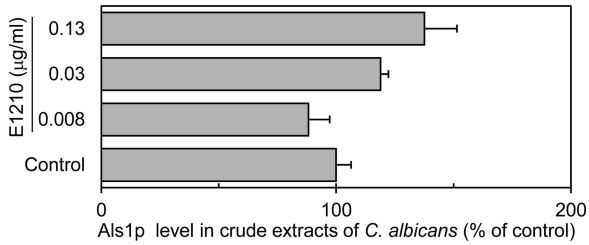


**FIG 3** Effects of E1210 on the Als1 protein level from crude extracts of *C. albicans* IFM49971 cells. The *C. albicans* cells treated with E1210 for 1 h were disrupted by mixing with glass beads, and the Als1 protein levels from crude extracts were determined utilizing ELISA and standardized using the protein concentration from crude extracts. Each data point represents the mean ± SEM ($n = 3$) from experiments performed in duplicate.

were not significant compared to the levels in the control cells. Furthermore, Rho1p expression on the surface of *C. albicans* IFM49971 cells grown for 1 h in MOPS–RPMI 1640 was also investigated. E1210 also showed no inhibition of Rho1p expression on the cell surface at concentrations tested (0.002 to 0.13 μg/ml) (Fig. 4). These results suggested that the suppression of Als1p expression on the cell surface by E1210 does not result from Als1p synthesis inhibition and that GPI biosynthesis inhibition of E1210 leads to impairment of the maturation or translocation of GPI-anchored proteins.

**Effect of E1210 on *C. albicans* adherence.** It was expected that E1210 could inhibit the adherence of *C. albicans* cells to polystyrene surfaces since E1210 inhibited the maturation of Als1p, an adhesin of *C. albicans*. *C. albicans* IFM49971 cells that were not incubated in MOPS–RPMI 1640 (0-h incubation) showed little adherence to polystyrene surfaces (2.9% ± 0.6% of the level in control cells grown in MOPS–RPMI 1640 for 1 h). Figure 5 shows the effects of E1210 and other antifungals on the adherence of *C. albicans* cells to a polystyrene surface after incubation for 1 h. The adherence rate [(number of adherent cells/number of inoculated cells) × 100] of untreated cells was 42.3% ± 3.1%. E1210 inhibited the adherence of *C. albicans* cells in a concentration-dependent manner with an $IC_{50}$ of 0.0039 μg/ml (95% CI, 0.0024 to 0.0063 μg/ml), which was lower than its MIC (0.008 μg/ml). No clumping was observed in nonadherent (planktonic) cells treated with E1210. Amphotericin B also inhibited *C. albicans* adherence in a concentration-dependent manner, but its $IC_{50}$ was 1.2 μg/ml (95% CI, 0.79 to 1.9 μg/ml), which was about 5-fold

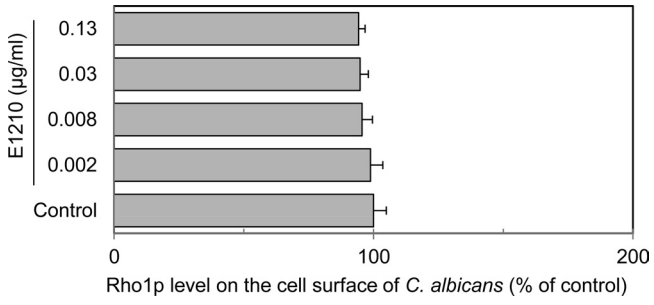


**FIG 4** Effects of E1210 on the Rho1 protein levels on the surface of *C. albicans* IFM49971 cells. The relative amounts of Rho1 protein on the cell surface of *C. albicans* treated with each compound in MOPS–RPMI 1640 medium for 1 h were determined on filter plates using ELISAs. Each data point represents the mean ± SEM ($n = 6$).

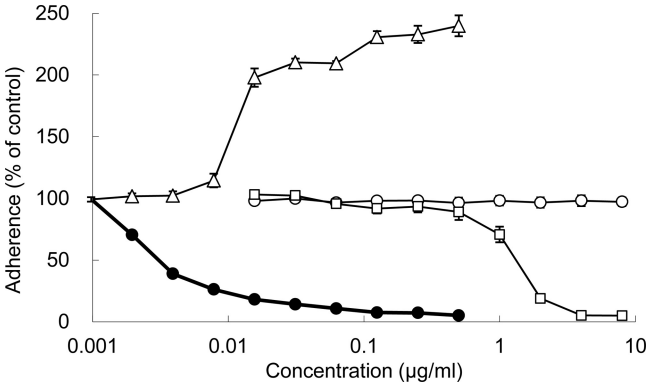


**FIG 5** Effects of E1210 and other antifungals on the adherence of *C. albicans* IFM49971 cells to polystyrene surfaces. *C. albicans* cells were incubated in the presence of each compound for 1 h in MOPS–RPMI 1640, and cells that adhered to polystyrene surfaces were stained using crystal violet. Each data point represents the mean ± SEM from three independent plates assayed in duplicate. Symbols: ●, E1210; ○, fluconazole; △, micafungin; □, amphotericin B.

higher than its MIC (0.25 μg/ml). Fluconazole showed little inhibition of *C. albicans* adherence within the range of concentrations tested (0.016 to 8 μg/ml). In contrast, micafungin enhanced *C. albicans* adherence about 2-fold at concentrations above its MIC (0.016 μg/ml). These results were consistent with the effects of these compounds on Als1p expression levels on the cell surface of *C. albicans*: E1210 reduced both Als1p expression on the cell surface and the adherence of cells to a polystyrene surface at concentrations above its MIC, while micafungin enhanced both, and fluconazole showed no effects.

**Effect of E1210 on germ tube formation.** The inhibitory effects of E1210 and other antifungals on germ tube formation in *C. albicans* IFM49971 cells were investigated (Fig. 6). E1210, micafungin, and amphotericin B inhibited germ tube formation by *C. albicans* with $IC_{50}$s of 0.0071, 0.015, and 0.24 μg/ml (95% CIs, 0.0054 to 0.0093, 0.014 to 0.016, and 0.20 to 0.29 μg/ml), respectively, each of which was close to the respective MIC. No inhibition of germ tube formation was seen with fluconazole at concen-

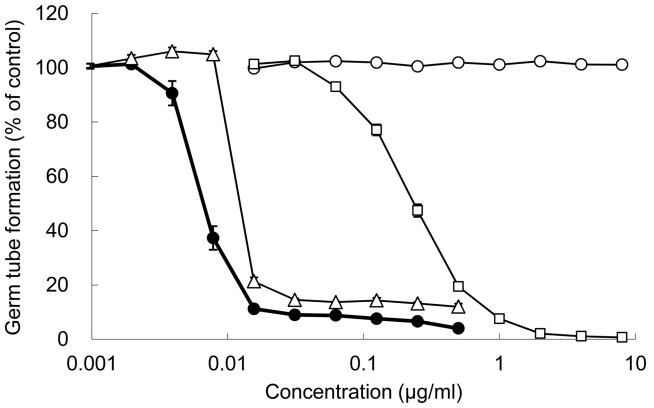


**FIG 6** Effects of E1210 and other antifungals on germ tube formation in *C. albicans* IFM49971 cells. *C. albicans* cells were incubated in the presence of each compound for 4 h in MOPS–RPMI 1640, and cells that converted from yeast form to the hyphal form and adhered to polystyrene surfaces were stained with crystal violet. Each data point represents the mean ± SEM from three independent plates assayed in duplicate. Symbols: ●, E1210; ○, fluconazole; △, micafungin; □, amphotericin B.

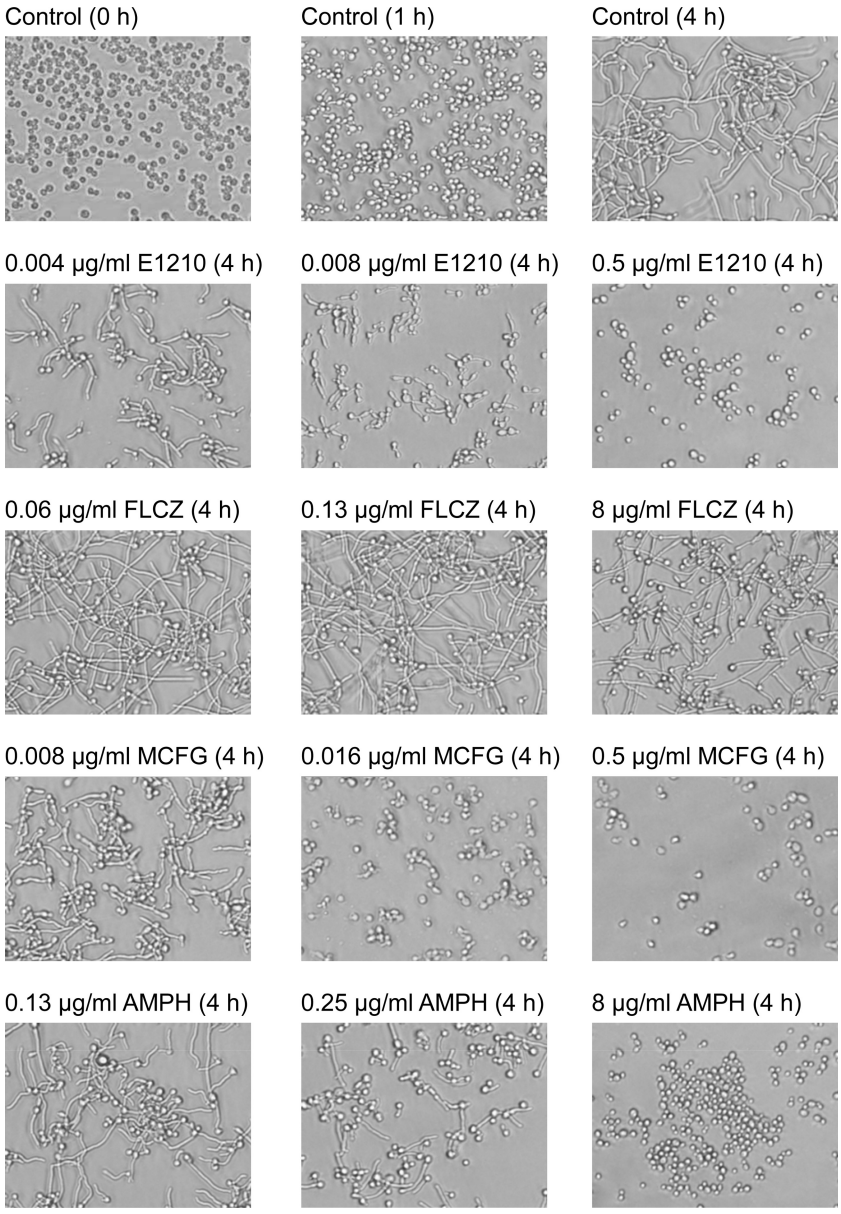


**FIG 7** Microscopic images of *C. albicans* cells after treatment with E1210 and other antifungals for 4 h. Cells were left untreated or were treated with 0.5×MIC, 1×MIC, and the highest concentration tested of each compound for 4 h. The magnification of all images is ×200. Abbreviations: FLCZ, fluconazole; MCFG, micafungin; AMPH, amphotericin B.

trations of ≤8 μg/ml. Figure 7 shows micrographs of *C. albicans* cultures after treatment with E1210 and other antifungals. Indeed, E1210, micafungin, and amphotericin B showed concentration-dependent inhibition of the yeast-to-hypha transition and showed marked inhibition of germ tube formation at each MIC (Fig. 7), whereas germ tube formation was observed even at a concentration of 8 μg/ml for fluconazole (Fig. 7). Although others have reported that azole antifungals can inhibit germ tube formation (20, 65), our observations were more consistent with those of Brayman and Wilks (5). These discrepancies might be due to the use of different growth parameters, media, and/or *C. albicans* strains.

Effects of E1210 on the hyphal development of *C. albicans* were also evaluated in other hypha-inducing media: Spider medium, Lee's medium, YPD supplemented with 10% FCS, and YNB supplemented with 2.5 mM GlcNAc (Fig. 8). E1210 suppressed the hyphal development of *C. albicans* in all media tested, but in Spider and Lee's media, some germinated cells were observed even at 4×MIC of E1210 (Fig. 9) and the inhibition of the yeast-to-hypha transition in both media was milder than in MOPS–RPMI 1640 (Fig. 8). Thus, E1210 inhibited the development of hyphal growth of *C. albicans*, but the compound also inhibited the yeast growth. The MIC of E1210 was 0.016 μg/ml for cells cultured in YNB, which does not induce the hyphal growth of *C. albicans*.

**Hyphal growth of *C. albicans* cells embedded in a matrix.** Since growth of *C. albicans* cells embedded within a matrix promoted the formation of hyphae (6), the morphology of cells growing in colonies in YPS agar medium was studied. Untreated cells

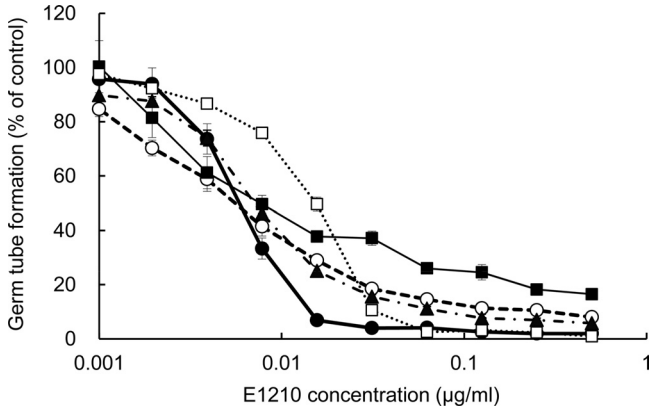


**FIG 8** Effects of E1210 on germ tube formation in *C. albicans* IFM49971 cells in various media. The *C. albicans* cells were incubated in the presence of E1210 for 4 h, and cells that converted from yeast form to hyphal form and adhered to polystyrene surfaces were stained with crystal violet. Each data point represents the mean ± SEM from three independent plates assayed in duplicate. Symbols: ●, MOPS–RPMI 1640; ○, Spider medium; ■, Lee's medium; □, YPD broth supplemented with 10% FCS; ▲, yeast nitrogen base without amino acids but supplemented with 2.5 mM GlcNAc.

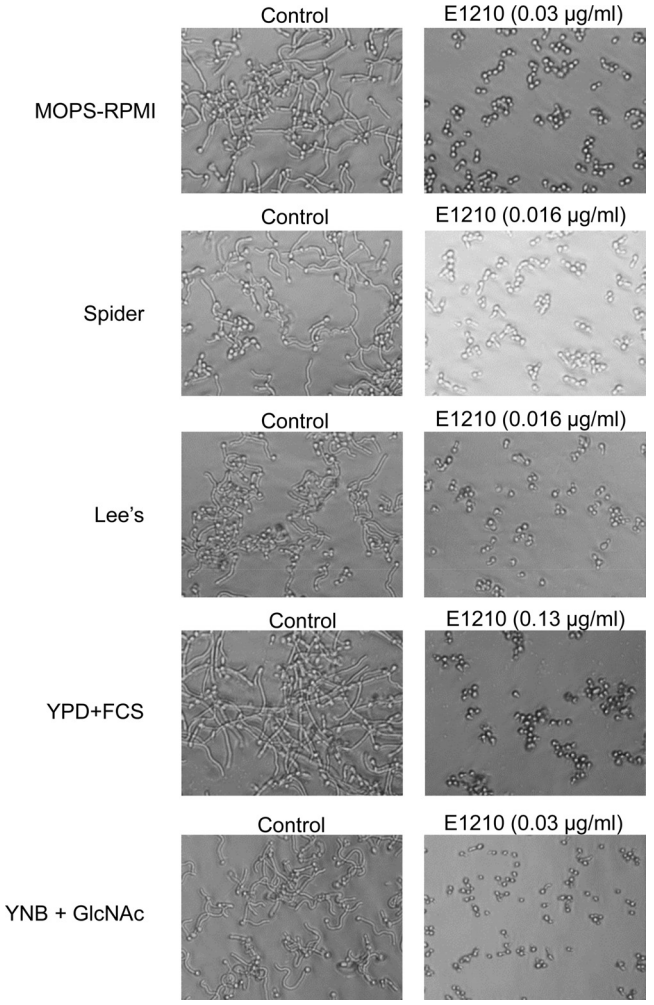


**FIG 9** Microscopic images of *C. albicans* IFM49971 cells after treatment with E1210 for 4 h in hypha-inducing media. Images of cells untreated or treated at 4×MIC of E1210 in each medium are shown. The magnification of all images is ×200. Abbreviations: YPD + FCS, YPD broth supplemented with 10% FCS; YNB + GlcNAc, yeast nitrogen base without amino acids but supplemented with 2.5 mM GlcNAc.

(control) showed hyphal or filamentous growth when embedded in YPS agar for 48 h. E1210 suppressed hyphal growth at concentrations of 0.002 μg/ml, but at 0.001 μg/ml, filamentous but not highly elongated cells were observed (Fig. 10). At 0.008 μg/ml (1×MIC), E1210 inhibited hyphal and colony growth.

**Biofilm formation.** *C. albicans* biofilms were developed in MOPS–RPMI 1640 supplemented with 10% FCS after incubation for 24 h. Biofilm formation by *C. albicans* was assessed by a biofilm density assay using safranin staining of extracellular polymeric materials that the biofilm cells produced. The MICs of E1210, fluconazole, micafungin, and amphotericin B for *C. albicans* IFM49971 in MOPS–RPMI 1640 supplemented with 10% FCS were 0.016, 0.25, 0.06, and 0.25 μg/ml, respectively. E1210 inhibited biofilm formation in a concentration-dependent manner with an $IC_{50}$ of 0.0044 μg/ml (95% CI, 0.0037 to 0.0051 μg/ml), which was 4-fold lower than its MIC, and almost entirely inhibited biofilm formation at a concentration of 0.008 μg/ml (Fig. 11). Micafungin and amphotericin B also inhibited *C. albicans* biofilm formation in a concentration-dependent manner, and their $IC_{50}$s were 0.014 μg/ml (95% CI, 0.0052 to 0.038 μg/ml) and 0.085 μg/ml (95% CI, 0.076 to 0.095 μg/ml), respectively. The inhibition curve of micafungin was not sigmoidal, and it inhibited biofilm formation even at the lowest concentrations tested. Fluconazole showed some inhibition of biofilm formation, with an $IC_{50}$ of 0.86 μg/ml (95% CI, 0.53 to 1.4 μg/ml), but did not completely inhibit biofilm formation even at the highest concentration tested (4 μg/ml), which was 16 times higher than its MIC.

*C. albicans* biofilms were observed by scanning electron microscopy (Fig. 12). The control (untreated) biofilms were composed of yeast, hyphal, and pseudohyphal elements; however, most of the exopolymeric materials (exopolysaccharides) were lost due to hydration during the procedures required for scanning electron microscopy (49). E1210-treated cells were less hyphal, and the scanning electron micrograph revealed that E1210 inhibited the development of biofilm formation even at half of its MIC.

Since E1210 inhibited biofilm formation in *C. albicans*, we investigated the antifungal activity of E1210 against sessile cells of *C. albicans*. SMICs of E1210 for the 36 strains of *C. albicans* tested ranged from 0.016 to 0.25 μg/ml (Table 2). The $SMIC_{50}$ and $SMIC_{90}$ of E1210 were 0.03 and 0.13 μg/ml, respectively, which were 4- to 8-fold greater than its $MIC_{50}$ and $MIC_{90}$ for planktonic cells. The activities of micafungin and amphotericin B against sessile cells were 8-fold lower than those against planktonic cells, but fluconazole's activity against sessile cells was >64-fold lower than the activity against planktonic cells. The *in vitro* activity of E1210 against sessile cells was almost the same as that of micafungin. These results suggested that E1210 should be active against *C. albicans* in biofilms.

## DISCUSSION

In yeasts, GPIs are transferred to selected glycoproteins that are then transported to the plasma membrane, where they remain anchored to the outer surface of the plasma membrane via their GPI or become cross-linked to the cell wall (26). Many proteins in the cell wall and plasma membrane involved in fungal cell wall

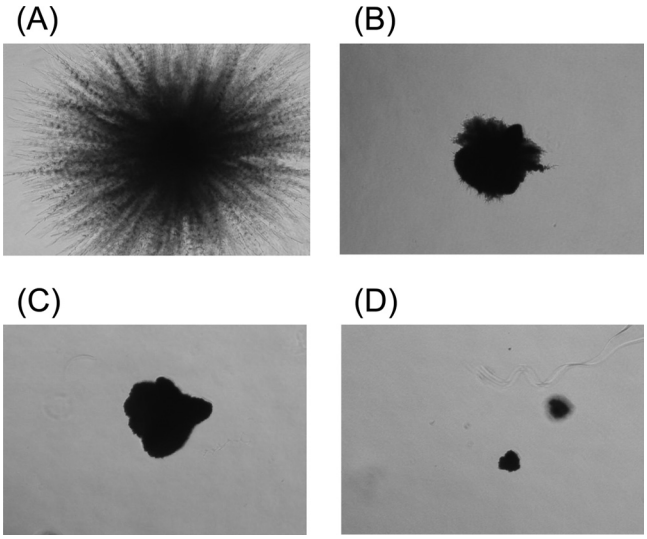


**FIG 10** Microscopic images of *C. albicans* colonies growing in YPS agar for 48 h. (A) Control; (B) 0.001 μg/ml E1210; (C) 0.002 μg/ml E1210; (D) 0.008 μg/ml E1210. Colonies were photographed at ×40.

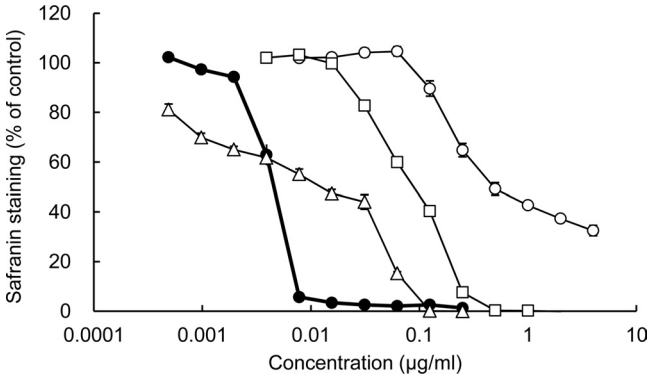


**FIG 11** Effects of E1210 and other antifungals on the density of *C. albicans* IFM49971 biofilms after incubation for 24 h. The biofilm density was quantified using safranin staining. Each data point represents the mean ± SEM from experiments on three independent plates assayed in duplicate. Symbols: ●, E1210; ○, fluconazole; △, micafungin; □, amphotericin B.

synthesis and assembly are GPI anchored, and GPI synthesis and/or anchoring is essential for cell viability in yeasts (27, 29, 43, 46). Although there is a GPI core structure (ethanolamine-P-6Man$\alpha$1-2Man$\alpha$1-6Man$\alpha$1-4GlcNH$_2\alpha$1-6-D-*myo*-inositol-1-phosphate-lipid, where the lipid is either diacylglycerol, acylalkylglycerol, or ceramide) which is conserved in all eukaryotes (26), many subtle but significant differences that can occur as the result of additional species-specific modifications to the structure and assembly of GPI have been reported, leading to the notion that these could each be exploited as targets for new antifungal drugs (15, 17). Some inhibitors of GPI biosynthesis have been reported (52, 57), but most of these are designed based on acceptor analogs or protease inhibitors and do not possess drug-like properties. Serine protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluoride inhibit ethanolamine phosphate addition and inositol acylation of GPI in *Toxoplasma gondii* and *Trypanosoma brucei*, which are suggested to have an activated serine that is absent in the other orthologues (56). YW3548, a natural product, is thought to inhibit the addition of phosphoethanolamine (EtNP) to the first mannose of GPI, but it inhibits both yeast and mammalian GPI-EtNP transferases, although EtNP modification of the first mannose is not essential for the surface expression of GPI-anchored proteins on mammalian cells (22, 58). We have modified the prototype compound BIQ (38, 59, 63) and developed E1210, which possesses potent antifungal activity (19, 34). In this study, E1210 was shown to inhibit *C. albicans* and *A. fumigatus* Gwt1ps, which are suggested to be involved in the inositol acylation of GlcN-PI in the GPI biosynthesis pathway (36, 63, 64), but not human Pig-Wp. In a large-scale survey of heterozygous mutants of *C. albicans* (69), Gwt1p was not a target of the existing antifungal compounds. This method could be useful for validating the target protein of E1210 in *C. albicans*. No inhibitors with sufficient fungal specificity and with properties that could lead to drug development have been reported to date. E1210 would be a first-in-class antifungal that acts by GPI biosynthesis inhibition.

Since most eukaryotes possess *GWT1* orthologous genes and since there are several homologous regions in each Gwp1p (63, 70), it is very interesting that E1210 possesses activity against Gwt1ps that is specific to fungi. Although the binding site of E1210 on fungal Gwt1ps remains unclear, E1210 might interact in part with the N-terminal end adjacent to the first homologous region of Gwt1ps, since a mutation in this region of ScGwt1p led to decreased susceptibility to BIQ (63). The differences in the amino acid sequence of this region between fungi and mammals might confer the species specificity of Gwt1p to E1210.

Virulence factors in *C. albicans* include proteins which mediate adherence to and invasion of host tissues, morphological changes from yeast to hyphae, secretion of lytic enzymes, maintenance of cell wall integrity, and avoidance of the host immune response (16). Some of these virulence factors are GPI-anchored proteins, which comprise 88% of all covalently linked cell wall proteins in *C. albicans* (25). Examples of GPI-anchored virulence factors are the Als protein family, adhesins with broad substrate specificity (15, 23, 55); Hwp1p, an epithelial adhesin and biofilm promoter (40); Eap1p, an adhesin to epithelial cells and polystyrene surfaces (30); secreted aspartyl proteinases Sap9p and Sap10p, phospholipase B Plb5p, and superoxide dismutases Sod4p, Sod5p, and Sod6p, which are also adhesins (2, 7, 32, 60). Numerous GPI-anchored proteins have been identified as virulence factors in *C. albicans*; however, non-GPI-anchored proteins are also virulence factors (21). Interactions involved with many proteins are necessary for pathogens to exhibit maximum virulence, but individual deletion of a number of proteins could alter the virulence phenotypes, such as adherence to surfaces and ligands. The compound that inhibits expression of one or more of these might affect surface function. As expected, E1210 suppressed the expression of Als1p on the cell surface of *C. albicans*, suggesting that E1210 inhibited GPI biosynthesis in *C. albicans*, resulting in the reduction of mature cell surface protein levels, which led to a reduction in the adherence potential of *C. albicans*, although this reduced adherence potential could not be due only to the reduced expression levels of Als1p. BIQ, a prototype Gwt1p inhibitor, also reduced the adherence of *C. albicans* to epithelial cells (63). Since E1210 inhibited GPI biosynthesis, it could be expected that the compound would lead to impaired expression of all GPI-anchored proteins. Further validation will be required. In contrast, micafungin, a glucan synthase

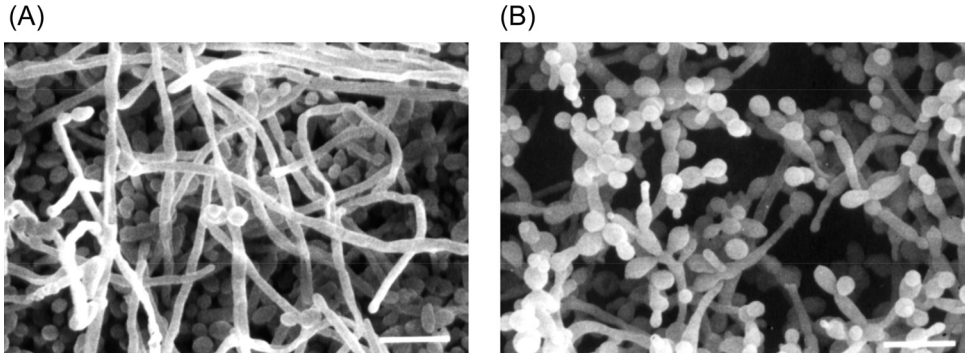


**FIG 12** Scanning electron micrographs of *C. albicans* IFM49971 biofilms in plastic slide wells after incubation for 24 h with either medium (A) or 0.008 μg E1210 per ml (B). Bar, 10 μm.

inhibitor, temporarily enhanced the Als1p levels on the cell surface and the adherence of *C. albicans* during a 1-h treatment. Since β-1,3-glucan biosynthesis is inhibited by micafungin, the cell surface protein levels might be increased so as to compensate for the osmotic pressure. However, micafungin is a fungicidal compound, and further treatment could lead to cell death.

Moreover, E1210 suppressed germ tube formation of *C. albicans* cells, indicating that E1210 could suppress hyphal growth of *C. albicans*. As the Als1p defect reduced adherence and hyphal growth of *C. albicans* (15, 55), GPI biosynthesis inhibition is expected to lead to the suppression of hyphal growth and reduce adherence of *C. albicans* to host or material surfaces. The yeast-to-hypha transition inhibition by E1210 seems to occur as the result not of signal transduction inhibition but of the maturation inhibition of GPI-anchored proteins involved in cell wall assembly, such as via exo-1,3-glucanase (Exg2 and Spr1), β-1,6-glucan synthase (Kre1), transglycosidase (Crh1), β-1,3-glucanosyltransferase (Phr1 and Phr2), and chitinase (Cht1 and Cht2) (7, 51). However, micafungin suppressed germ tube formation even though it increased Als1p levels. The germ tube formation inhibition caused by micafungin is thought to result from inhibition of glucan chain extension (5).

The expression of adhesins and the dimorphism of *C. albicans* have each been reported to be associated with biofilm formation (3, 41, 49). E1210, which inhibited Als1p expression and germ tube formation, indeed showed substantial inhibition of biofilm formation. E1210 was as active against *C. albicans* in biofilms as micafungin and amphotericin B, which are fungicidal agents against *C. albicans*. The antifungal activity of E1210 is fungistatic (34), but E1210 may be expected to indirectly kill *Candida* cells in hosts, since the deficiency of GPI reduced the resistance of *C. albicans* to macrophages (50).

Toenjes et al. (61) and Brayman and Wilks (5) have proposed that the yeast-to-hypha transition assay or germ tube formation assay should be used to identify signal transduction inhibitors or glucan synthase inhibitors. Since E1210 inhibits germ tube formation in *C. albicans*, the yeast-to-hypha transition assay might be a good tool for the identification of GPI biosynthesis inhibitors.

Thus, E1210 was suggested to inhibit the GPI biosynthesis, leading to the suppression of *C. albicans* hyphal growth, adherence, and biofilm formation. The GPI biosynthesis pathway has been investigated mainly in *S. cerevisiae*, but there are a few reports on *C. albicans* GPI, and some *C. albicans* mutants lacking the genes involved with GPI biosynthesis show contrariety in some phenotypes. The *GPI7*-null mutant showed reduced hyphal growth on solid media, increased sensitivity to Calcofluor white (CFW), reduced virulence in mice, and lytic action of macrophages (50). The *PGA1* mutant showed reduced adherence and biofilm formation and increased sensitivity to CFW but increased filamentation (18). The conditional null mutation of *GPI19* led to reduced surface GPI anchor levels, cell wall biogenesis aberrations (aggregation), and increased filamentation, but not increased CFW sensitivity (67). The conditional null mutant of *SMP3* exhibited reduced surface GPI anchor levels, aberrant morphology (aggregation of irregularly shaped cells), and increased CFW sensitivity

**TABLE 2** Comparative susceptibilities of planktonic and sessile forms of 36 *C. albicans* strains

| | No. of strains with MIC (μg/ml) of: | | | | | | | | | | | | | | | MIC (μg/ml) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Compound[a] | ≤0.002 | 0.004 | 0.008 | 0.016 | 0.03 | 0.06 | 0.13 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | >16 | 50% | 90% |
| Planktonic | | | | | | | | | | | | | | | | | |
| E1210 | 1 | 8 | 24 | 3 | | | | | | | | | | | | 0.008 | 0.016 |
| FLCZ | | | | | | 3 | 27 | 4 | | | | | | | 2 | 0.13 | 0.25 |
| MCFG | | 5 | 28 | 2 | 1 | | | | | | | | | | | 0.008 | 0.016 |
| AMPH | | | | | | | 26 | 8 | 2 | | | | | | | 0.13 | 0.25 |
| Sessile | | | | | | | | | | | | | | | | | |
| E1210 | | | | 5 | 15 | 11 | 4 | 1 | | | | | | | | 0.03 | 0.13 |
| FLCZ | | | | | | | | | | | 1 | | | | 35 | >16 | >16 |
| MCFG | | | | 2 | 6 | 24 | 3 | | 1 | | | | | | | 0.06 | 0.13 |
| AMPH | | | | | | | | | 2 | 31 | 3 | | | | | 1 | 2 |

[a] Abbreviations: FLCZ, fluconazole; MCFG, micafungin; AMPH, amphotericin B.

(17). Although GPI biosynthesis inhibition was not confirmed in the *PGA1* mutant, it is surprising that each mutant unable to product mature GPI shows a different phenotype. In addition, from a large-scale survey of homozygously null mutants, *GPI8* was suggested to be essential (11), whereas a null mutant of *GPI11* was viable (13), although both orthologous genes of *S. cerevisiae* are essential. Although the *GWT1/gwt1* mutant showed a reduced growth rate, reduced hyphal development, and reduced virulence in mice, a *gwt1/gwt1* null mutant was not obtained (unpublished data). This was probably because the *GWT1* gene is essential in *C. albicans*. E1210 could exhibit growth inhibition through the GPI biosynthesis inhibition if so. Further studies on GPI biosynthesis of *C. albicans* are needed.

Moreover, the majority of surface proteins in protozoans, such as *T. brucei* VSG, whose analysis led to the discovery of the GPI anchor, are GPI anchored, and GPI defects lead to lethality in these parasites, especially *T. brucei* (14, 37). Since inositol acylation of GlcN-PI is a prerequisite for mannosylation and other subsequent modifications in other protozoans, such as *Plasmodium falciparum*, E1210 or a derivative of it could be active against these protozoans. However, mannosylation precedes inositol acylation in *T. brucei*, and inositol acyltransferase of *T. brucei* is inhibited by serine protease inhibitor PMSF and GlcN-(2-*O*-alkyl) PI analogs, which do not inhibit the inositol acyltransferases of mammals and fungi (52, 57, 52). *Trypanosoma* might possess inositol acyltransferases different from those of other eukaryotes. The activity of Gwt1p inhibitors against protozoans should therefore also be studied.

The antifungal characteristics of E1210 are fascinating, and further studies leading to its clinical development, including mode-of-action studies, are warranted.

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